AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

STATUTORY DECLARATION

I, Kari Alitalo of The Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, SF-00014 Helsinki, Finland do solemnly and sincerely declare as follows :

Introduction

1. Background

1.1 I am presently working as Research Professor with The Finnish Medical Research Council of the Finnish Academy of Sciences. Since receiving my M.D. and M.Sc.D. in 1977 and 1980, respectively, from the University of Helsinki, I have worked substantially continuously as a professor and scientific researcher in Finland in areas of cellular and molecular biology and cancer research. My research has included substantial studies and explorations in fields of cancer, cancer metastasis, angiogenesis, lymphangiogenesis, and other areas related to angiogenesis. In addition to my own research efforts and my collaborations with others, I receive numerous invitations to speak at national and international symposiums in these areas of study, I supervise post-graduate research of others, I have authored and co-authored numerous original research articles published in peer-reviewed journals, and I have served on the editorial board of such journals. My detailed *curriculum vitae* is attached hereto as Exhibit 1.

1.2 I have conducted and collaborated in substantial research relating to a growth factor gene and protein that my laboratory calls "Vascular Endothelial Growth Factor C" or "VEGF-C." My attached *curriculum vitae* shows that I have co-authored several publications in peer-reviewed journals relating to the VEGF-C gene and protein, its synthesis and processing in cells, and its biological activities *in vitro* and *in vivo*. Among these publications are the following:

> **Document D70**: Joukov et al., "A Novel Vascular Endothelial Growth Factor, VEGF-C, Is a Ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) Receptor Tyrosine Kinases," *EMBO J., 15(2)*: 290-298 (1996).
>
> **Document D71**: Joukov et al., "Proteolytic Processing regulates receptor specificity and activity of VEGF-C," EMBO J., 16(13): 3898-3911 (1997)
>
> **Document D74**: Kukk et al., "VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development," *Development, 122*: 3829-37 (1996).

I also have filed patent applications relating to VEGF-C, VEGF-C variants, and uses thereof. Among these applications are the following applications:

> **Document D72**: International Patent Application No. PCT/FI96/00427, filed on 1 August 1996 by Helsinki University Licensing Ltd Oy (WO 97/05250).
>
> **Document D73**: International Patent Application No. PCT/US98/01973, filed on 2 February 1998 by Ludwig Institute for Cancer Research et al. (WO 98/33917).

Thus, my laboratory and my collaborators have substantial expertise and experience working with and expressing the VEGF-C gene and protein.

1.3 I am familiar with the opposition filed by Ludwig Institute for Cancer Research ("Ludwig Institute") to the issuance of a patent to Human Genome Sciences, Inc., ("HGS") based on HGS's Australian Patent Application No. 696764 ("the opposed application"). Ludwig Institute asked me to perform a protein expression study that may be relevant to the opposition, and provide this declaration in which I report the study and the results.

1.4 In making this declaration to the Australian Patent Office, I understand that I have an overriding duty to the Patent Office (and to any Australian Federal Court that should review the Patent Office decision) to provide objective scientific analysis that I believe to be truthful. I hereby affirm that, to the best of my knowledge and belief, factual statements herein are true and opinion statements herein represent my objective scientific opinion and analysis.

II. VEGF2 and VEGF-C

2.1 The human growth factor which my laboratory and others in the scientific community call "VEGF-C" is encoded by a human gene having 419 codons. The coding sequence of a VEGF-C cDNA may be found in **Document D73** or in the publicly accessible Genbank database under Accession No. X94216.

2.2 The 350 amino acid VEGF-2 polypeptide sequence disclosed in the opposed application of Human Genome Sciences, entitled "Vascular Endothelial Growth Factor 2" (VEGF-2(HGS)) corresponds to amino acid residues 70 to 419 of human VEGF-C (Genbank Accession No. X94216), with the exception of a single amino acid difference (Lys/Gln) at position 414 of the VEGF-C sequence.[1] HGS subsequently filed a later patent application that contained a 419 amino acid "full length" VEGF2 sequence. (See, e.g., Fig 1A-1E of **Document D44** (WO 96/39515)) The 419 residue VEGF-C and VEGF2 sequences are identical except for two amino acid differences: one at position 3 (Leu/Ser), and another at position 414 (Lys/Gln) of the VEGF-C sequence. Thus, my experience working with VEGF-C is applicable to working with VEGF2.

III. Signal Peptides

[1] The opposed patent application actually contains sequence ambiguities. If one compares the VEGF-C sequence with the VEGF2 sequence in the Sequence Listing of the opposed application, one observes amino acid differences at residue 73 and 414, and an insertion of an extra Cys residue in the VEGF2 sequence at a location between residues 369 and 370 of the 419 residue VEGF-C sequence. Based on HGS's later filed patent applications, I have concluded that the VEGF2 sequences in the figures were more appropriate to use in the experiments described herein.

3.1 Polypeptides such as growth factors that are destined for extracellular secretion are first synthesized in the cellular cytoplasm. Such polypeptides generally include a short secretory signal peptide at their amino terminus that is usually cleaved off, but serves as a vital signal to direct the nascent polypeptide into the cell's protein secretion apparatus.

3.2 Scientific experiments in my laboratory has determined that the first approximately 31 amino acids from the 419 amino acid form of VEGF-C serve as a signal peptide. The experimental details and evidence underlying this determination are reported in **Document D71.**

3.3 In the opposed patent application, the 350 amino acid VEGF2 sequence is lacking the 31 amino acids that represent the VEGF-C signal peptide. In the application, the inventors assert that the first 24 amino acids of their VEGF2 sequence (which would approximately correspond to amino acids 70-93 of the full-length 419 amino acid VEGF-C sequence) operate as a signal peptide.

Experimental Purpose

4.1 In view of my laboratory's expertise in expressing and working with the VEGF-C gene and protein, the Ludwig Institute asked me to perform experiments to determine whether or not the 350 amino acid protein contains an operative signal peptide, as alleged in the opposed application.

Experimental Design

I. Overview

5.1 The accumulated knowledge of molecular biologists regarding signal peptides have permitted biologists to identify certain characteristic features of signal peptides. (One such feature is an amino acid composition comprising largely hydrophobic residues.) Computer programs have been designed to predict whether an amino acid sequence begins with a signal peptide, and to identify the site in an amino acid sequence where a putative signal peptide is cleaved. As a first part of my analysis, I used one such

program, the SignalP program at the Center for Biological Sequence Analysis, The Technical University of Denmark, to analyze the approximately 350 amino acid VEGF2 sequence for a series of residues having characteristics of a signal sequence.

5.2. As a second part of my analysis, I transformed a mammalian cell line with an expression vector containing a polynucleotide that encodes the 350 amino acid VEGF2 sequence ("VEGF2(HGS)"), grew the cell line under conditions in which the cells produce polypeptides, and then assayed the growth medium of the cells to determine whether the cells were secreting VEGF2. These experiments included various experimental controls to assure that there was no problem with the expression vector, the cells, the transformation procedures, the growth conditions, or other parameters. The actual details of the experimental protocol are described in the next section.

II. Detailed Experimental Protocol

6.1 To determine whether eukcaryotic cells can express and secrete VEGF2(HGS), an expression plasmid containing a VEGF2(HGS) polynucleotide sequence was constructed. This involved preparing a VEGF2(HGS) DNA fragment, and inserting the fragment into a commercial expression vector.

6.1.1 The polymerase chain reaction (PCR) was employed to construct a DNA fragment that encodes amino acids 70 to 419 of VEGF-C, followed by a short hemagglutinin (HA) tag fused in-frame to the 3' end of the VEGF-C coding region.[2] The 5'-primer used in the PCR reaction contained a BamHI restriction endonuclease recognition site followed by the first 18 nucleotides from the VEGF-C(70-419) coding sequence. The 3'-primer contained an XbaI recognition

[2] As explained above, amino acids 70-419 of VEGF-C differ at position 414 from the VEGF2(HGS) amino acid sequence presented in the figures of the opposed patent. Since any signal peptide in VEGF2(HGS) would occur at the *beginning* (amino terminus) of the VEGF2(HGS) sequence, a single change at position 414, and the inclusion of a HA-tag at the end (carboxy terminus) are inconsequential to this expression study. These assumptions are verified by the VEGF-C positive control that was included in these experiments, and by the ability of my laboratory and many other laboratories to recombinantly express other polypeptides with a carboxy terminal HA tag to facilitate purification.

site, an HA-tag, a stop codon, and the last 15 nucleotides from the VEGF-C(70-419) coding region, excluding the stop codon. The locations of the 5' and 3' primers with respect to the complete VEGF-C cDNAsequence (which was used as PCR template DNA), are shown in Exhibit 2 attached hereto.

6.1.2 The resulting PCR product was digested with BamHI and XbaI and inserted into the multiple cloning site of the commercially available expression vector pcDNA1/Amp (Invitrogen) that had been digested with the same enzymes. This construct was named VEGF2(HGS)/pcDNA1, and DNA sequencing was performed to confirm that the VEGF2(HGS) insert was present and in the correct orientation for expression.

6.1.3 To serve as an experimental control, a similar expression plasmid, designated VEGF-C/pcDNA1 was also constructed. In this expression plasmid, a DNA encoding the complete 419 amino acid VEGF-C polypeptide was cloned into pcDNA1.

6.2 The 293T mammalian cell line was selected for the expression study. Thus, 293T cells, grown in DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin/streptomycin, were mock-transfected (control), transiently transfected with VEGF2(HGS)/pcDNA1, or transiently transfected with VEGF-C/pcDNA1 using the calcium-phosphate method.

6.3 Radioactive amino acids that would be incorporated into nascent polypeptides were introduced into the cell growth medium to assist in the identification of expressed polypeptides. In particular, 48 hours after transfection, the transfected cells were washed twice with phosphate-buffered saline (PBS) and metabolically labeled in MEM medium containing 100μCi/ml ^{35}S-methonine and ^{35}S-cysteine (Promix, Amersham) for 6 hours. The conditioned media was harvested and cleared of contaminants by centrifugation. After washing three times with ice cold PBS, the cells were lysed in ice cold RIPA-buffer

(150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris); supplemented with 0.01 U/ml aprotinin, 1μg/ml leupeptin, and 1mM PMSF; and the lysate was cleared by centrifugation.

6.4 Before analysis for expressed VEGF2(HGS) and VEGF-C, steps were taken to assure that any low levels of VEGF produced by 293T cells would not confound the results. Endogenous VEGF was removed from the conditioned media and cell lysates by incubation with 1 μg/ml monoclonal anti-human VEGF antibody (R & D Systems), followed by precipitation of the immunocomplexes with protein A-Sepharose (Amersham Pharmacia Biotech).

6.5 Next, an immunoprecipitation was conducted to capture any VEGF2(HGS) or VEGF-C from the conditioned media or cell lysates. For immunoprecipitation, the conditioned media was supplemented with BSA, Tween 20, and heparin to final concentrations of 0.5%, 0.02%, and 1 μg/ml, respectively. VEGF2(HGS) was immunoprecipitated at 4 C with 4 μg/ml monoclonal anti-HA antibody (HA.11, BabCO), and VEGF-C was immunoprecipitated at 4 C with 882 antiserum, a polyclonal antibody raised against a synthetic peptide corresponding to residues 35-51 of the 350 amino acid VEGF2 polypeptide. The immunocomplexes were collected on protein A-Sepharose and washed twice with 1X binding buffer (0.5% BSA, 0.02% Tween 20, 1 μg/ml heparin), and once with 20 mM TrisHCl pH 7.4 at 4 C. The proteins were analyzed on 15% SDS-PAGE under reducing conditions.

Experimental Results

7.1 Analysis of the VEGF2(HGS) sequence with the SignalP program indicated that this 350 amino acid sequence does not begin with a sequence having hydrophobicity characteristics of a signal sequence.

7.2 An autoradiogram of the SDS-PAGE gel is attached hereto as Exhibit 3. That exhibit shows that the VEGF2(HGS) polypeptide is detected in cell lysates (lane 4), but not

conditioned media (lane 1), from 293T cells transfected with VEGF2(HGS)/pcDNA1. In contrast, VEGF-C polypeptide was detected in both cell lysates (lane 5) and conditioned media (lane 2) from 293T cells transfected with VEGF-C/pcDNA1. VEGF2(HGS) detected in cell lysates migrates as a circa 46 kD protein, whereas the majority of VEGF-C detected in the conditioned media migrated as a broad doublet band of approximately 29-31 kD polypeptides and another band of about 21 kD. A significant quantity of higher molecular weight polypeptides were observed in the cell lysates of the VEGF-C-transfected cells, which I interpret as VEGF-C "captured" at various stages of proteolytic processing[3] (as a result of lysing the cells six hours after labeling. In addition, it is readily apparent from the autoradiogram that the expression level of VEGF-C is much higher than that of VEGF2(HGS).

Analysis

8.1 If VEGF2(HGS)-transfected cells had secreted any VEGF2(HGS) protein, the protein would have been captured by the anti-HA antibody and visualized in the conditioned medium from these cells (Exhibit 3, lane 1). No VEGF2(HGS) was observed in this lane, indicating that no VEGF2(HGS) secretion was occurring. Thus, I conclude that the 350 amino acid VEGF2 sequence taught in the opposed application does NOT contain a signal peptide sequence. This conclusion is further supported by the computer analysis which failed to identify any sequence in the 350 residue VEGF2 that has hydrophobicity characteristics of a signal peptide.

8.2 The experimental procedures were sound, as evinced by the high level of secreted VEGF-C that was observed in the conditioned media of cells that had been transfected with the full-length VEGF-C cDNA construct (lane 2), and the observation of a well-defined, unsecreted 46 kD polypeptide band captured by the anti-HA antibody from the cell lysate of VEGF2(HGS)-transfected cells.

[3] A detailed description of VEGF-C proteolytic processing is set forth in **Document D71**, which I incorporate herein by reference.

8.3 The fact that VEGF-C expression observable in cell lysates of VEGF-C-transfected cells is much higher than VEGF2(HGS) expression observable in VEGF2(HGS)-transfected cells suggests that VEGF2(HGS) is inefficiently translated and/or that the intracellular turnover rate of VEGF2(HGS) is much faster than that of VEGF-C. In other words, the cells may be recognizing VEGF2(HGS) as an aberrant protein and rapidly degrading it.

Summary

9.1 The failure of cells transfected with an expression vector containing the 350 amino acid VEGF2 cDNA sequence taught in the opposed patent application to secrete any VEGF2 protein indicates that the 350 amino acid VEGF2 cDNA sequence taught in the opposed application does not contain a functional signal peptide, as the patent applicants allege.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

DECLARED at Helsinki

this 15th day of February 2000

Kari Alitalo

BEFORE ME:

OLLI-PEKKA SIRO
Notary Public
Notary Public